REMARKS/ARGUMENTS

With this amendment, claims 1-8, 16, and 28-29 are pending. Claims 9-15 and 17-27 are cancelled. For convenience, the Examiner's rejections are addressed in the order presented in the March 22, 2006 Office Action.

I. Rejections under 35 U.S.C. §112, first paragraph, written description

According to the Office Action, claims 1-8, 16, 28 and 29 are rejected because the claims contain subject matter that was allegedly not described in the specification in a manner to convey that the inventors had possession of the claimed invention at the time of filing. The Office alleges that the claims introduce new matter. The Office Action further alleges that the claimed antibodies are subgenus of antibodies that was "not contemplated in the specification as originally filed." Applicants respectfully traverse the rejection. Both the recited antigen, *i.e.*, SEQ ID NO:68, and antibodies that bind to at least one epitope of that antigen were clearly contemplated and disclosed in the originally filed specification.

The specification discloses both a genus of frizzled extracellular domains and also calls out each individual member of that genus at, *e.g.*, the original claims. Original claim 10 depends from original claim 1, and thus includes all the limitations of claim 1, including, *e.g.*, "wherein said antibody binds to at least one epitope in an extracellular domain of the frizzle receptor. . ." Original claim 10 further recites that the frizzled receptor extracellular domain is 80% homologous to an amino acid sequence selected from a group of SEQ ID NOs: 61, 62, 63, 64, 66, **68**, 69, 71, 73, 75, and 77. Thus, original claim 10 provides disclosure and proof of contemplation of each recited individual frizzled extracellular domain. The Office Action does not provide any reasoning to suggest that a list of individual species fails to support disclosure of each species individually.

The description of the Fzd 5 extracellular domain provided in SEQ ID NO:68 is a fully characterized antigen and therefore, provides description the antibodies that selectively bind to the antigen. The Office Action does not provide any reasoning that disclosure of a fully characterized antigen is not sufficient to disclose antibodies that selectively bind to the antigen.

Moreover, original claim 1 makes clear that that contemplated antibodies bind to any of the epitopes in a frizzled extracellular domain, *i.e.*, to "at least one epitope."

In the previous response, Applicants provided evidence that original claims 1 and 10 provide support for antibodies against the frizzled 5 extracellular domain that inhibit growth of a malignant cell. Those arguments are maintained, but are not repeated in this response.

In view of the above arguments, withdrawal of the rejection for alleged lack of written description is respectfully requested.

II. Rejections under 35 U.S.C. §103(a)

Claims 1-8, 16 and 28-29 are rejected under 35 U.S.C. §103(a) as allegedly obvious over of Tanaka *et al.* in view of US Patent No. 5,677,171 (Hudziak *et al.*). Applicants respectfully traverse. The Office Action has not established a *prima facie* case of obviousness. To establish a *prima facie* case of obviousness, three basic criteria must be met: (1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) there must be a reasonable expectation of success; and (3) the prior art reference must teach or suggest all the claims limitations. MPEP§2143. See also *In re Rouffet,* 47 USPQ2d 1453. The court in *Rouffet* stated that "even when the level of skill in the art is high, the Board must identify specifically the principle, known to one of ordinary skill, that suggests the claimed combination." *Rouffet* at 1459. The court has also stated that actual evidence of a suggestion, or teaching, or motivation to combine is required and the showing of a suggestion, or teaching, or motivation to combine must be "clear and particular." *In re Dembiczak*, 50 USPQ2d 1614, 1617 (1999).

The claims are directed to antibodies that bind to the amino terminal extracellular domain of the frizzled 5 receptor, and that inhibit growth of a malignant cell that expresses the frizzled 5 protein. The claimed invention is based, at least in part, on the recognition that frizzled proteins, including frizzled 5, are overexpressed in some cancers, and thus, can be used as tumor specific antigens that can be used to generate immunotherapy agents. The claimed antibodies are immunotherapy agents used to inhibit growth of or kill cancer cells. None of the

cited references provide evidence that the frizzled 5 protein is overexpressed in malignant cells or that antibodies directed against frizzled 5 are useful to kill cancer cells that express frizzled 5. As such, the claimed antibodies are a patentably distinct species of the broad genus of Frizzled 5 antibodies, referred to by the Office Action. Without recognition of the role of frizzled proteins in cancer, antibodies against frizzled proteins would be raised only to function as research tools, not as growth-inhibiting, immunotherapeutic agents as required by the claims. In addition, none of the cited references disclose the specifically claimed amino terminal extracellular domain of frizzled 5 or antibodies against that domain.

Tanaka *et al.* disclose the cloning of the frizzled 7 gene and report that frizzled 7 is overexpressed in esophageal cancer. Tanaka *et al.* disclose a portion of frizzled 5 amino acid sequence (not the same as SEQ ID NO:68) and further show that expression of frizzled 5 is not correlated with esophageal cancer (see, *e.g.*, Figure 1). Thus, Tanaka *et al.* fail to disclose the claimed amino terminal extracellular frizzled 5 sequence and, in fact teach away from a role for frizzled 5 in cancer. Because of that failure, the disclosure of Tanaka *et al.* also fail to provide evidence of a motivation to identify antibodies directed against frizzled 5 that inhibit proliferation of cancer cells.

The Office Action alleges that Tanaka *et al.* discloses that ectodomains of frizzled proteins function as a "natural antagonist" of frizzled mediated signal transduction and that frizzled proteins are receptors for "Wnt oncoproteins". Thus, the Office Action assumes that all wnts and frizzled proteins have assigned functions. This is not correct. First, Tanaka *et al.* at page 10164 do not refer to Wnt oncoproteins, but rather refer to Wnt proteins that are ligands for Fzd proteins. Thus, unlike the Office Action, Tanaka *et al.* do not suppose a general role for all Wnt proteins in tumorogenesis. Applicants submit as Exhibit A Wong *et al.* Mol. Cell. Biol. 14:6278 (1994), which discloses that wnt function was unsettled at the time of filing. Wong *et al.* transfected a mammary epithelial cell line with expression plasmids for each of nine wnt proteins, including wnt 5a, to determine each proteins ability to transform mammalian cells. At page 6280, right column, Wong *et al.* disclose that, unlike some wnt proteins, the wnt5a protein had no transforming activity and thus, is categorized as a poorly transforming or non-

transforming wnt protein. The Office Action provides no evidence that wnt5 or its receptor are involved in tumorigenesis.

The Office Action also cites Hudziak *et al.* as disclosing antibodies that bind to extracellular ligand binding sites on receptors for growth factors and that inhibit growth of tumor cells. Hudziak *et al.* were, in fact, very selective in their choice of growth factor receptor antibodies. Hudziak *et al.* selected the HER2 protein. The related HER2 gene was known to be overexpressed in mammary carcinoma cell lines and overexpression of the HER2 protein in the non-transformed NIH 3T3 cell line transformed the NIH 3T3 cells. The HER2-overexpressing NIH 3T3 cells caused tumor formation when injected into nude mice, a classic test for oncogenic properties of an expressed protein. Thus, in order to generate antibodies that inhibited growth of malignant cells, Hudziak *et al.* carefully selected the product of the HER2 gene as an antigen, relying on the well-characterized and convincingly demonstrated oncogenic properties of the HER2 gene and protein. Hudziak *et al.* do not suggest that any protein with an extracellular ligand binding domain is a suitable candidate to raise antibodies that will inhibit the growth of malignant cells. Rather Hudziak *et al.* guides those of skill to select protein antigens that have demonstrated tumor promoting properties.

Hudziak *et al.* provide no motivation or expectation of success that antibodies against fzd5, a protein with no demonstrated link to tumor formation, would inhibit growth of malignant cells. Unlike HER2, frizzled 5 was not shown to be overexpressed in a naturally occurring transformed cell line and was not shown to have transforming ability if artificially overexpressed in a nontransformed cell line. The frizzled 5 receptor had none of the oncogenic properties that characterize HER2 and guided selection of HER2 as an antigen to produce antibodies that inhibit growth of a malignant cell line. Tanaka *et al.* did not disclose any oncogenic properties associated with the Frizzled 5 protein or any reason for those of skill to suspect that the frizzled 5 protein would have oncogenic properties. Thus, even an attempt to combine the cited references to arrive at, *i.e.*, antibodies directed to the extracellular binding domain of the non-oncogenic frizzled 5 protein, would not provide a motivation or reasonable expectation of success in generating antibodies that inhibit growth of a malignant cell. Therefore, the combination of cited references does not render the claimed antibodies obvious.

In view of the above arguments, withdrawal of the rejections for alleged obviousness is respectfully requested.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

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Differential Transformation of Mammary Epithelial Cells by *Wnt* Genes

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The mouse *Wnt* family includes at least 10 genes that encode structurally related secreted glycoproteins. *Wnt-1* and *Wnt-3* were originally identified as oncogenes activated by the insertion of mouse mammary tumor virus in virus-induced mammary adenocarcinomas, although they are not expressed in the normal mammary gland. However, five other *Wnt* genes are differentially expressed during development of adult mammary tissue, suggesting that they may play distinct roles in various phases of mammary gland growth and development. Induction of transformation by *Wnt-1* and *Wnt-3* may be due to interference with these normal regulatory events; however, there is no direct evidence for this hypothesis. We have tested *Wnt* family members for the ability to induce transformation of cultured mammary cells. The results demonstrate that the *Wnt* gene family can be divided into three groups depending on their ability to induce morphological transformation and altered growth characteristics of the C57MG mammary epithelial cell line. *Wnt-1*, *Wnt-3A*, and *Wnt-7A* were highly transforming and induced colonies which formed and shed balls of cells. *Wnt-2*, *Wnt-5B*, and *Wnt-7B* also induced transformation but with a lower frequency and an apparent decrease in saturation density. In contrast, *Wnt-6* and two other family members which are normally expressed in C57MG cells, *Wnt-4* and *Wnt-5A*, failed to induce transformation. These data demonstrate that the *Wnt* genes have distinct effects on cell growth and should not be regarded as functionally equivalent.

The *Wnt-1* proto-oncogene was originally identified as a common integration site of mouse mammary tumor virus in independently isolated adenocarcinomas of mammary epithelial tissue (31). Ectopic expression of the normally silent *Wnt-1* locus results from the introduction of transcriptional enhancers contained in the mouse mammary tumor virus long terminal repeats (30, 31). Formal proof of a causative role for *Wnt-1* in mammary oncogenesis has come from experiments on gene transfer into mammary epithelial cell lines (5, 41) and transgenic mice (50).

In C57MG cells, an epithelial cell line derived from normal mouse mammary tissue (51), *Wnt-1* expression causes a morphological transformation (5, 18). Normal C57MG cells grow in a monolayer with a regular, cuboidal appearance at confluence; *Wnt-1* expression causes the cells to become refractile and elongated, growing over other cells in a disorganized pattern. However, this transformation is considered only partial, since the morphologically changed cells are unable to develop tumors in syngeneic host animals. In contrast, a second cell line, RAC311C, derived from a mammary tumor which has lost its tumorigenic capacity is fully transformed by *Wnt-1* exhibiting morphological transformation in vitro and tumor formation in vivo (41). In transgenic mice (50) and in retrovirally infected reconstituted mammary glands (10), ectopic *Wnt-1* expression has been shown to result in enhanced proliferation of mammary epithelial cells in virgin females. In summary, these experiments demonstrate that *Wnt-1* induces morphological transformation of mammary epithelial cell lines and hyperplasia in the virgin mammary gland, suggesting that *Wnt-1* acts by disrupting normal growth regulation.

In addition to *Wnt-1*, *Wnt-2* and *Wnt-3* also have transforming activity. *Wnt-2* has been shown to morphologically transform C57MG cells (1). *Wnt-3* was initially identified as an oncogene activated in tumors arising from mouse mammary tumor virus insertion (42). Thus *Wnt-1*, *Wnt-2*, and *Wnt-3* appear to be functionally related in their ability to disrupt normal growth regulation in mammary epithelium.

The *Wnt* gene family has now grown to at least 15 vertebrate members (reviewed in references 26 and 32), 10 of which have been identified in the mouse. However, little is known about their biological properties. Comparison of the predicted amino acid sequence from the *Wnt* genes indicates that they encode cysteine-rich proteins with an average amino acid identity of 50%, although three pairs (*Wnt-3/3A*, *Wnt-5A/5B*, and *Wnt-7A/7B*) which probably arose from more recent gene duplication events show between 80 and 90% identity. The amino terminus invariably encodes a typical hydrophobic signal sequence. Moreover, the gene products of *Wnt-1*, *Wnt-2*, and the *Drosophila Wnt-1* ortholog, the segment polarity gene *wingless* (*wg*) (40), have been shown to be secreted (1, 4, 14, 22, 37, 52). Thus, it is likely that all Wnt proteins are secreted and mediate interactions between cells. *Wnt-1* is also glycosylated at four N-linked glycosylation sites, although glycosylation does not appear to be essential for transformation in culture (22).

Direct observation of wg protein in the *Drosophila* embryo (14) and biochemical analysis of Wnt-1 (18) in mammalian cell culture indicates that Wnt signalling is probably quite local, acting over only a few cell diameters. This narrow range in activity is most likely due to retention of secreted Wnt protein on the cell surface or within the extracellular matrix (1, 4, 37, 52). Unfortunately, difficulties encountered in biochemically analyzing Wnt signalling have hindered studies on the signalling pathway. For example, no receptor has been identified though it seems likely that Wnt signalling occurs through a conventional receptor-mediated process triggered by binding of Wnt ligand.

Until recently it was unclear why the mammary gland is a target site for Wnt-mediated transformation, especially as the

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EXHIBIT A

three transforming *Wnt* genes thus far identified, *Wnt-1*, *Wnt-2*, and *Wnt-3*, are not normally expressed in the mammary gland. Recent studies indicate that six *Wnt* family members, *Wnt-4*, *Wnt-5A*, *Wnt-5B*, *Wnt-6*, *Wnt-7B* (11), and *Wnt-2* (6), are expressed and developmentally regulated in the mammary gland. Thus *Wnt* genes may be involved in the normal regulation of mammary growth and development, and it is one or more of these endogenous signalling pathways which is likely to be deregulated during ectopic expression of *Wnt-1*, *Wnt-2*, or *Wnt-3*. This may result from a simple overexpression of a proliferative signal with properties that resemble those of one of the endogenously expressed Wnt proteins, causing a hyperstimulation of a proliferative pathway. Alternatively, the transforming *Wnt* genes may interfere with potentially growth-suppressing or differentiating signals mediated by one or more of the *Wnt* genes normally expressed in the mammary gland.

To explore the issue of *Wnt* signalling and its relationship to normal and transforming activity in the mammary gland, we have studied the transforming activities of all available mouse *Wnt* genes in the C57MG mammary epithelial cell line. We demonstrate that the *Wnt* gene family can be divided into three groups based on the ability to morphologically transform C57MG cells in vitro. Interestingly, the *Wnt* genes *Wnt-4* and *Wnt-5A*, which are normally expressed in the mammary gland and in the C57MG cell line, are incapable of inducing morphological transformation and deregulated growth. This finding demonstrates that transformation is not due to simply overexpressing a Wnt protein. In contrast, *Wnt-5B* and *Wnt-7B*, which are also expressed in the developing mammary gland, are moderately transforming, equal to *Wnt-2*. These results suggest that not all Wnt proteins are functionally equivalent and, moreover, that Wnt-mediated hyperplasia in vivo may result from inappropriate activation of a *Wnt-5B* and/or *Wnt-7B* signalling pathway.

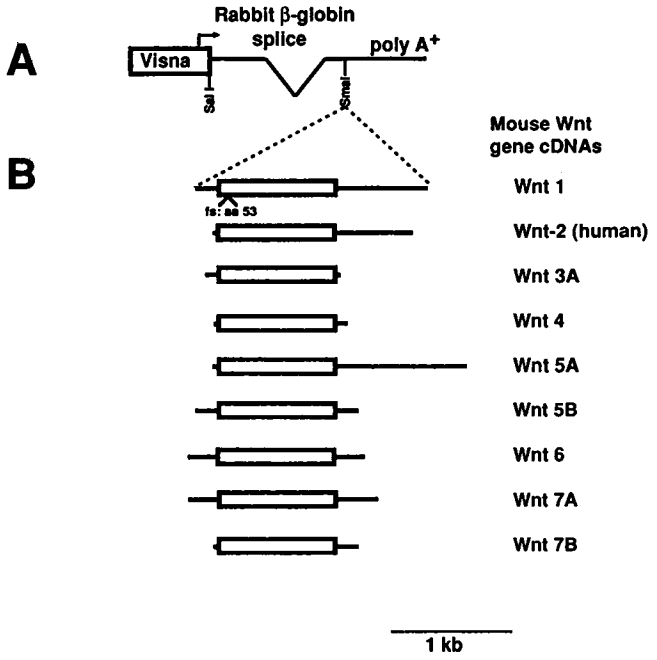

FIG. 1. Map of *Wnt* gene expression constructs used to transform C57MG cells in vitro. (A) Map of vector pBLsoVISInpA. The vector includes a visna virus promoter, splice donor, splice acceptor, and intron II of rabbit β-globin, and the polyadenylation site from simian virus 40. (B) Map of *Wnt* gene cDNAs cloned into the *Sma*I site of the vector. Each *Wnt* gene cDNA insert, including the full protein-coding region, some 5′ transcribed leader, and some 3′ untranslated region, was purified and recloned into the *Sma*I cloning site of pBLsoVIS InpA. These expression clones were linearized with either *Pvu*I or *Nde*I and cotransfected with PGKneobpA into C57MG cells. fs, frameshift; aa, amino acid.

MATERIALS AND METHODS

Construction of recombinant genes. cDNA inserts containing complete protein-coding regions, translational initiation sites, and termination sites of several mouse *Wnt* genes were previously cloned (12) and assembled to generate contiguous, complete cDNA inserts. A full-length human *Wnt-2* cDNA was used for these experiments (55). As a negative control, a frameshift was created in *Wnt-1* by end filling a unique *Xho*I site (encoding amino acid position 53), using the Klenow fragment of DNA polymerase I. Each insert was excised and blunt end ligated into the *Sma*I site of pBL-soVISInpA (Fig. 1). pBL-soVISInpA (provided by Craig Rosen) contains a 650-bp *Xho*I-*Sal*I fragment containing a visna virus promoter cloned upstream of the 1-kb *Sal*I-*Bgl*II rabbit β-globin intron II and simian virus 40 polyadenylation region. Plasmid DNA used for transfection into cultured C57MG cells was purified by CsCl gradients and linearized with *Pvu*I or *Nde*I prior to transfection.

Cell culture and transfections. C57MG cells were grown in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin, and 10 mg of bovine pancreatic insulin (Sigma) per ml. Calcium phosphate transfections were performed by using a mammalian transfection kit (Stratagene) on 20% confluent dishes of cells. Each visna virus-*Wnt* construct was cotransfected with pPGKneobpA (46) digested with *Xho*I, in a molar ratio of 5:1. Subsequent growth of stably transformed colonies for the focus-forming assay was in the presence of 1.2 mg of Geneticin per ml. After 14 to 20 days of selection, transformed colonies were clearly visible, and plates were scored for morphological transformation.

Growth in serum-free conditions was performed with individual clones or pooled plates of stable transfections. Cells were plated into six-well tissue culture dishes in duplicate in DMEM and 10% fetal calf serum and then allowed to settle and adhere overnight. The medium was then changed to HB-CHO basal salt medium (Irvine Scientific) with 10 mg of bovine pancreatic insulin per ml (22). Cells were allowed to grow 1 week past confluence, with a change of the HB-CHO medium every 3 days, and the cell cultures were then photographed.

RNA purification. Individual clones picked from dishes of transformed colonies after 2 to 3 weeks of selection were expanded, checked by PCR for the transfected DNA, and grown to confluence in triplicate 15-cm-diameter dishes in complete DMEM. Cells were briefly rinsed in cold phosphate-buffered saline, lysed in RNAzol B, and scraped from the dishes. RNA was purified essentially by the method of Chomczynski and Sacchi (7).

Construction of RNase protection probes and RNase protection assays. A fragment of each visna virus-*Wnt* gene construct containing sequences from the rabbit β-globin intron upstream of the splice acceptor site, from either the *Sca*I site (293 bases 5′ of the splice acceptor site) or the *Apa*I site (42 bases 5′ of the splice acceptor site) through the splice acceptor and into sequences of each *Wnt* cDNA, was subcloned. The amount of sequence contributed from each *Wnt* gene was as

follows: from *Wnt-1* up to the *Pvu*II site at position 210, from *Wnt-3A* from the *Bss*HII site at position 100 up to the *Pvu*II site at position 270, from Wnt-4 to the *Stu*I site at position 237, and from *Wnt-5A* from the *Eco*RI site at position 459 up to the *Sma*I site at position 549. These DNA restriction fragments were blunt end ligated into the *Eco*RV site of pBSt II KS+ and sequenced with both T3 and T7 primers. The *Wnt-4* probe fragment was cloned into *Apa*I-*Eco*RV sites of pGEM7Zf+.

To generate radiolabeled RNase protection probes, each template DNA was linearized at a unique restriction enzyme site at the 5′ end and transcribed in the presence of [^{32}P]UTP (as described in Promega's "Protocols and Applications Guide"). The transcription reactions were DNase I treated, and RNA probes were gel purified on 5% urea–acrylamide gels. RNase A-plus-T_1 protections were performed with the Ambion RPA II kit as described by the manufacturer. The control mouse β-actin probe provided in the kit was transcribed with the other probes as described. RNase protection was performed on 40 μg of total RNA per sample, as measured by optical density. Each hybridization was done with 5×10^4 cpm of labeled *Wnt* RNA and 10^3 cpm of control actin RNA. The protected RNA products of RNase A-plus-T_1 digestion were separated on 5% urea–acrylamide gels and sized relative to ^{32}P-labeled *Msp*I-digested pBR322 DNA markers. Gels were dried onto Whatman 3M paper and exposed to film to visualize the protected fragments.

Quantitation of RNase protection assays was performed with a Molecular Dynamics densitometer and ImageQuant software. Integrated volumes of each band, summing the intensities of all pixels in a defined band, was performed for the analysis shown in Table 2.

RESULTS

Morphological transformation of mammary epithelial cells by *Wnt* genes. To compare the transforming potential of each *Wnt* gene, expression constructs were transfected into the C57MG mouse mammary epithelial cell line. The parental C57MG cell line was originally derived from a normal retired breeder female C57BL/6 mouse (51) and grows as a contact-inhibited monolayer with simple squamous epithelial morphology (Fig. 2A). With the introduction of an actively expressed oncogene such as *Wnt-1*, these cells change cell shape and growth characteristics (5) (Fig. 2B). Transformed cells appear smaller, elongated, and refractile, growing very densely into chord-like bundles of cells or ball-forming colonies which often break off and float freely in the media.

All expression constructs containing the murine *Wnt* genes were transfected into C57MG cells, selected for G418 antibiotic resistance conferred by the neomycin phosphotransferase (*neo*) gene, and scored for morphological transformation. Table 1 presents the data accumulated from three independent cotransfection experiments in which the number of morphologically transformed colonies was scored out of the total number of neomycin resistant (Neo^r) colonies. Transfection of pPGKneobpA in the absence of an exogenous oncogene demonstrated a low level (5.1%) of spontaneous morphological transformation. However, individual clones of these cells reverted to the flat, simple squamous epithelial appearance of the parental cell line when the cells were grown in serum-free conditions (see below).

Three murine *Wnt* genes, *Wnt-1*, *Wnt-3A*, and *Wnt-7A*, demonstrated a high efficiency of morphological transformation, with 35.6, 41.3, and 40.0%, respectively, of all Neo^r colonies displaying the transformed phenotype (Table 1). Introduction of a frameshift mutation in the *Wnt-1* coding region at amino acid position 53 reduced transformation efficiency to 3.9%, approximately the same value observed for spontaneous transformation of PGKneobpA-transfected cells. Thus, morphological transformation was due to the expression of the *Wnt-1* proto-oncogene rather than the introduction of an active visna virus promoter.

Wnt-2, *Wnt-5B*, and *Wnt-7B* transformed C57MG cells at lower efficiencies of 19.1, 23.7, and 27.8%, respectively (Table 1). These colonies were smaller and more contact inhibited than those resulting from *Wnt-1* transformation. They also appeared morphologically less transformed (forming balls at lower frequency), suggesting an intermediate morphology in serum-containing media.

Wnt-4 and *Wnt-6* demonstrated little or no ability to transform C57MG cells, with efficiencies of 12.0 and 12.7%, respectively. *Wnt-5A* repeatedly was unable to demonstrate any ability to transform C57MG cells above background levels observed with the PGKneobpA alone (Table 1).

These experiments suggested that *Wnt* genes have different capacities to transform mammary epithelial cells. The highly transforming *Wnt* genes included *Wnt-1*, *Wnt-3A*, and *Wnt-7A*. A moderately transforming group of *Wnt* genes included *Wnt-5B* and *Wnt-7B*. A poorly transforming or nontransforming group of *Wnt* genes included *Wnt-4*, *Wnt-5A*, and *Wnt-6*.

Growth of *Wnt*-transformed C57MG cells in serum-free conditions. To test the transforming activity more rigorously, morphologically transformed colonies from each transfection were isolated and grown in serum-free defined medium. The elimination of serum from cell culture suppressed the background of spontaneously transformed colonies observed when untransfected C57MG cells are grown in the presence of serum (22).

In HB-CHO basal salt medium, C57MG cells remain mitotically active until reaching confluence and then stop dividing. The cells retain the appearance of a simple squamous epithelium and remain contact inhibited (Fig. 2A). However, *Wnt-1*-transformed C57MG cells grown in this defined medium maintain the morphology and growth characteristics of the transformed state (Fig. 2B). Cells are no longer contact inhibited and continue to divide past confluence. Examples of maintained morphological transformation in the absence of serum components for *Wnt-1*, *Wnt-3A*, and *Wnt-7A* are illustrated in Fig. 2B, E, and J.

Wnt-5B- and *Wnt-7B*-transfected C57MG cells, when grown in the absence of serum, demonstrated an intermediate morphology, growing with an apparently increased cell density after confluence but remaining contact inhibited (Fig. 2H and K). However, *Wnt-4*-, *Wnt-5A*-, and *Wnt-6*-transfected cells, while exhibiting a transformed phenotype in the presence of serum, reverted to the morphology of the parental cell line, flattening out on the plastic substrate and growing as a monolayer indistinguishable from untransfected cells. In addition, no growth past confluence in HB-CHO medium was observed (Fig. 2F, G, and I).

The results from this stringent assay of *Wnt* transformation clearly demonstrated that the *Wnt* genes could be separated into a highly transforming group including *Wnt-1*, *Wnt-3A*, and *Wnt-7A*, an intermediate group including *Wnt-5B* and *Wnt-7B*, and a nontransforming group including *Wnt-4*, *Wnt-5A*, and *Wnt-6*.

Expression of the *Wnt* genes in C57MG cells. The apparent differences in transforming activity of Wnt proteins may simply reflect differences in the level of *Wnt* gene expression. In the absence of specific Wnt antisera, we addressed this issue by examining the levels of *Wnt-1*, *Wnt-3A*, *Wnt-4*, and *Wnt-5A* transcripts in transfected C57MG cells by RNase protection

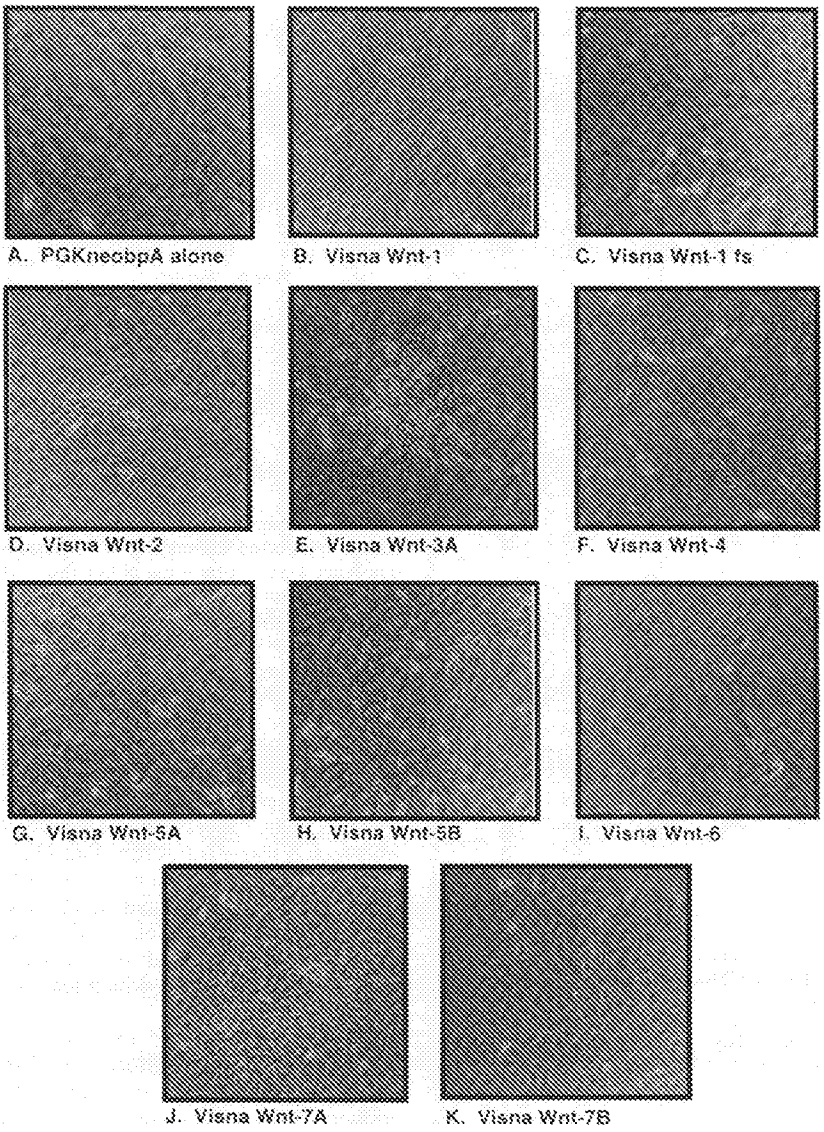

FIG. 2. Morphological phenotype of transfected C57MG cells grown in serum-free conditions. Individual transformed colonies were picked from each transfection, trypsinized, replated into duplicate wells of six-well dishes (Costar), and allowed to adhere overnight. Twenty-four hours later, the medium was changed in one set of duplicates to HB-CHO basal salt medium (Irvine Scientific) supplemented with insulin. Cells were allowed to grow 1 week past confluence, with a change of the medium every 3 days, and photographed. fs, frameshift.

(Fig. 3). *Wnt-4* and *Wnt-5A* were chosen because both were shown to be nontransforming in the transfection assay. Moreover, both *Wnt-4* and *Wnt-5A* are normally expressed by C57MG cells, indicating that they are not normally transforming when expressed at the endogenous levels in these cells (11).

RNase protection assays were performed on RNAs purified from pooled dishes of transfected C57MG cells rather than isolated colonies. These pools represented at least 100 independent Neo^r colonies and thus should represent average *Wnt* expression within the population of transfected colonies rather than expression per morphologically transformed cell. This was important since some of the transfected genes did not result in morphological transformation. Since the selection of these cells was only for Neo^r and not for morphological transformation, the ectopic expression of *Wnt* genes in these samples should also represent a conservative estimate of the actual levels of expression which may be obtained in individual colonies. Mouse β-actin was selected as an internal control to standardize levels of *Wnt* gene expression. The results of these experiments are shown in Fig. 4 and Table 2.

A 612-nucleotide (nt) *Wnt-1* probe protected a predicted fragment of 323 nt in *Wnt-1*-transfected C57MG cells (B in Fig. 4, lanes 4, 5, 13, and 14) but not in untransfected cells (lanes 7, 8, 10, and 11). In contrast, a mouse β-actin control probe protected the expected band of 250 nt in both transfected and untransfected C57MG cells (A in lanes 3, 4, 6, 7, 9, 10, 12, and 13). The relative levels of *Wnt-1* expression normalized to the actin level were 37 and 8% of the actin control level in two independent experimental pools. Similarly, the 597-nt *Wnt-3A* probe protected a predicted fragment of 308 nt only in *Wnt-3A*-transfected C57MG cells (C in Fig. 4, lanes 19 and 20; a smaller *Wnt-3A*-specific protected fragment represents some anomaly of the protection assay). *Wnt-3A* RNA expression

TABLE 1. Efficiency of transformation of C57MG cells by *Wnt* genes[a]

DNA	Expt	Total no. of colonies	No. transformed	% Transformed	Avg %
PGKneobpA	1	196	12	6.0	5.1
	2	43	2	4.6	
	3	106	5	4.7	
Visna virus–*Wnt-1*	1	115	54	47.0	35.6
	2	43	12	28.0	
	3	56	18	32.0	
Visna virus–*Wnt-1fs*	1	79	3	3.7	3.9
	2	53	2	3.8	
	3	49	2	4.1	
Visna virus–*Wnt-2*	1	117	23	19.6	19.0
	2	57	10	17.5	
	3	81	16	19.8	
Visna virus–*Wnt-3A*	1	196	80	41.0	41.3
	2	107	41	32.5	
	3	45	23	50.5	
Visna virus–*Wnt-4*	1	102	18	18.0	12.0
	2	85	10	11.8	
	3	47	3	6.1	
Visna virus–*Wnt-5A*	1	72	6	9.5	6.6
	2	57	3	5.0	
	3	55	3	5.4	
Visna virus–*Wnt-5B*	1	122	42	34.0	23.7
	2	98	17	17.5	
	3	61	12	19.5	
Visna virus–*Wnt-6*	1	86	11	12.7	12.7
	2	64	9	14.0	
	3	52	6	11.5	
Visna virus–*Wnt-7A*	1	56	19	33.5	40.0
	2	92	43	49.5	
	3	59	21	36.0	
Visna virus–*Wnt-7B*	1	24	5	21.0	27.8
	2	51	18	35.0	
	3	44	12	27.3	

[a] Three independent experiments were performed in which each *Wnt* expression construct and a *Wnt-1* frameshift mutation (visna virus–*Wnt*-1fs) as a negative control were cotransfected into C57MG cells with PGKneobpA. Input PGKneobpA DNA was held at 2 μg, while *Wnt* expression constructs were introduced at a 5:1 molar excess. Transfected cells were placed in selective Geneticin-containing medium for 14 to 20 days, and then plates were scored for total number of Geneticin-resistant colonies and total number of transformed colonies.

varied from 12% to as low as 0.9% of the endogenous actin control level in two experiments (Table 2).

In *Wnt-4*-transfected C57MG cells, the 492-nt *Wnt-4* RNase protection probe generated two specific products, a predicted 305-nt product (D in Fig. 4, lanes 25 and 26) and a smaller 270-nt product (E in lanes 25 and 26). Both products appeared to be specific for *Wnt-4*-transfected C57MG cells (lanes 25 and 26). The smaller fragment consistently appeared in the presence of the larger predicted protected fragment, despite alteration of the conditions of the RNase protection assay (data not shown). Since it was possible that this smaller product represented an alternative spliced transcript from the rabbit β-globin intron in the expression vector using an unexpected splice acceptor site in the *Wnt* gene sequence, reverse transcriptase PCR was performed to examine potential splice variants. With 5′ primers from either just upstream of the splice donor or just downstream of the splice acceptor in the rabbit β-globin sequence and a 3′ primer within the *Wnt-4* gene sequence, both primer pairs detected amplification products indistinguishable in size (data not shown). Thus, splice variants are not present, and the smaller protected fragment appears to be generated from the expected *Wnt-4* transcript produced by the expression cassette as a result of some artifact of the RNase protection procedure.

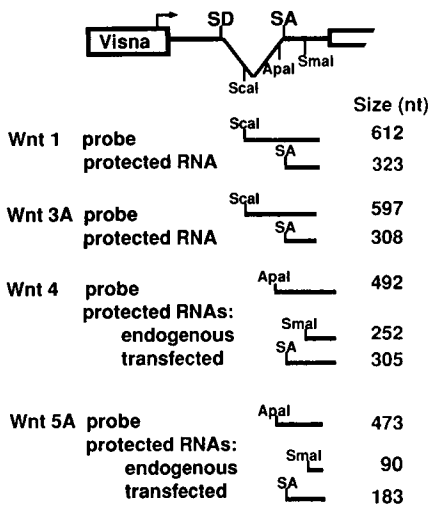

FIG. 3. Map of RNase protection assay probes used and predicted sizes of protected fragments. DNA fragments from each transfection construct were cut by using either the *Sca*I or *Apa*I site in the rabbit β-globin intron through the splice acceptor (SA) to a unique site within the *Wnt* cDNA. The sizes of the probes are presented as well as the sizes of the predicted protected products of RNase protection. SD, splice donor.

Interestingly, though *Wnt-4* is normally expressed by C57MG cells, expression is extremely low compared with the transfected *Wnt-4* transcript. The 252-nt endogenous protected product (Fig. 4, lane 23) is barely visible, while the transfected *Wnt-4* transcript is present at 16 to 47% of the levels of the actin control. Thus, levels of *Wnt-4* expression which do not result in morphological transformation are comparable to those of *Wnt-1* and *-3A*, which lead to efficient transformation (Table 3).

RNase protection of total RNA purified from *Wnt-5A*-transfected or nontransfected C57MG cells generated two *Wnt-5A*-specific products: a small 90-nt product representing endogenous *Wnt-5A* gene expression (G in Fig. 4, lanes 28, 29, 31, 32, 33, 34, 37, and 38) and the predicted 183-nt product of the transfected gene (F in lanes 31, 32, 37, and 38). Densitometry analysis revealed that both endogenous and transfected genes were expressed at approximately equivalent levels (Table 2), suggesting that a twofold increase in *Wnt-5A* expression is insufficient for morphological transformation of C57MG cells.

DISCUSSION

The experiments in this study have tested and compared the potential of each member of the *Wnt* gene family to induce transformation of cultured mouse mammary epithelial cells. *Wnt-1* and *Wnt-3A* caused relatively strong morphological changes in C57MG cells when expressed at levels which varied from as low as 0.9% to as high as 37% of an actin control level. There are at least two possible explanations for such highly transforming potential. Since there is no endogenous expression of *Wnt-1* or *Wnt-3A* in C57MG cells, it is possible that transformation is due to ectopic expression of genes not normally expressed in these cells. Presumably, the products of these ectopically expressed genes would aberrantly interact with putative receptors present on the cell surface which

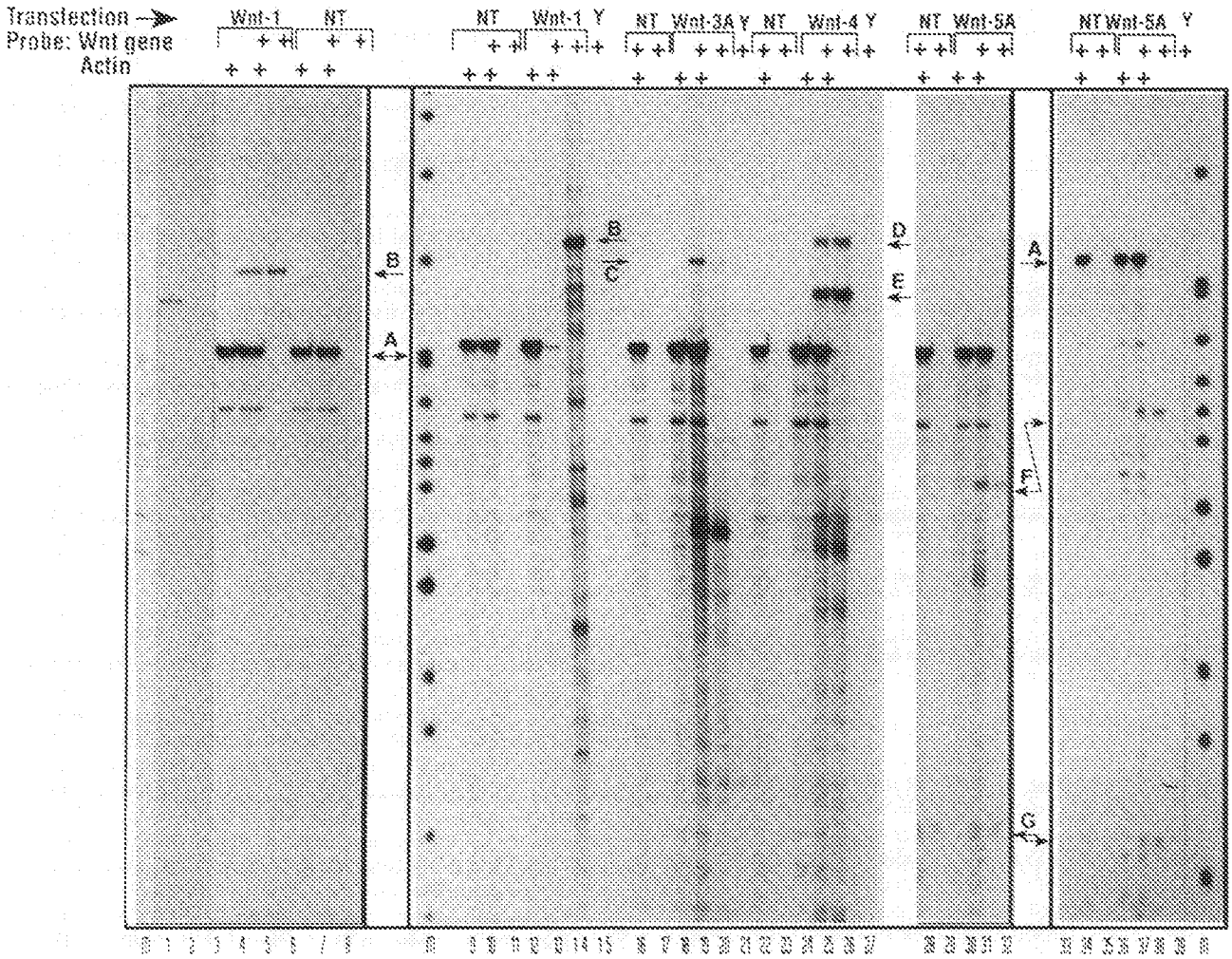

FIG. 4. RNase protection analysis of *Wnt* gene expression in transfected C57MG cells. Forty micrograms of total RNA from untransfected C57MG cells (NT) or transfected cells (as indicated) was hybridized with either 5×10^4 cpm of ^{32}P-labeled *Wnt* probe or 10^3 cpm of β-actin probe overnight at 55°C and then treated with RNases A and T_1 as described in Materials and Methods. Products are identified as follows: A, actin; B, Wnt-1; C, Wnt-3A; D and E, Wnt-4 (see text); F, transfected Wnt-5A; and G, endogenous Wnt-5A. Y indicates 20 μg of yeast RNA hybridized with each *Wnt* probe. Lanes 1 and 2 contain full-length (untreated with RNases A and T_1) actin and *Wnt-1* probes. Marker lanes are pBR322 digested with *Msp*I. For orientation, the doublet in marker lanes comigrating with the actin protected product represents 242 and 238 nt.

normally interact with the endogenously expressed Wnt ligands. This would result in a deregulated growth signal to which cells respond by phenotypic changes in cell morphology and growth characteristics as shown here. Alternatively, the highly transforming *Wnt* genes may be expressed at significantly higher levels than the endogenous genes, resulting in an overwhelming level of ligand expression which would cause a similar aberrant interaction with the endogenous putative receptors. In this second model, the levels of expression would be the critical aspect of the high transforming ability of *Wnt-1* and *Wnt-3A*. It was therefore of critical interest to determine if the nontransforming *Wnt-5A* and *Wnt-4* genes are expressed at levels comparable to that of the highly transforming members of the gene family.

The data shown in Fig. 4 and Table 2 demonstrate that *Wnt-4* expression from the transfected gene, as measured by densitometry, exceeded the levels of expression of either *Wnt-1* or *Wnt-3A*. This result suggests that high levels of expression of some *Wnt* genes may not be sufficient for morphological transformation of C57MG cells, although we cannot address whether there are differences in the translational efficiency of different RNAs in our experiments. Rather, transformation appears to depend upon the interaction of specific *Wnt* gene products with the putative receptor(s) on the cell surface of these cells. In agreement with this hypothesis, *Wnt-1*- and *Wnt-3A*-transformed cells varied greatly in the expression of the transfected genes but did not vary in resultant morphological transformation. These data are consistent with a hypothesis that these gene products may not encode equivalent signals or that signalling requires interactions with specific receptor molecules, some of which may not be present on the surface of C57MG cells. For example, C57MG cells may not express a *Wnt-4* and a *Wnt-5A* receptor, but in the presence of such a receptor, these two family members may transform. This conclusion is also consistent with the observations that many members of the *Wnt* gene family are normally expressed during specific stages of mammary gland development and differentiation as well as in the C57MG cell line (11), suggest-

TABLE 2. Quantitation of RNase protection assays by densitometric analysis

Transfected gene	Probe	Expt	Wnt/actin ratio[a]	Transfected product/endogenous product ratio[b]
Wnt-1	*Wnt-1*	1	0.37	
		2	0.08	
Wnt-3A	*Wnt-3A*	1	0.12	
		2	0.01	
Wnt-4	*Wnt-4* (endogenous)		—[c]	
	Wnt-4 (transfected)		0.16	6.9
Wnt-5A	*Wnt-5A* (endogenous)	1	0.21	
	Wnt-5A (transfected)		0.24	1.17
	Wnt-5A (endogenous)	2	0.13	
	Wnt-5A (transfected)		0.18	1.33

[a] In each RNA sample, the protected product from β-actin probe was assigned a value of 1. Quantitative comparison of *Wnt* protected product, as measured by Integrated Volume analysis (ImageQuant) and corrected for size, is expressed as a fraction of the actin product. Densitometry was performed from X-ray films shown in Fig. 4, lanes 2 and 13 (*Wnt-1*), 19 (*Wnt-3A*), 25 (*Wnt-4*), and 31 and 37 (*Wnt-5A*), in which the β-actin and *Wnt* probes were present in the same sample.

[b] In RNA samples from *Wnt-4* and *-5A* transfections, the protected product resulting from hybridization to the endogenously expressed *Wnt* gene was assigned a value of 1. Quantitative comparison of transfected *Wnt* protected product, corrected for size, is expressed as a fraction of endogenous Wnt product. Densitometry was performed on same lanes as listed above.

[c] The RNase protection product predicted for endogenous Wnt-4 (a product of 252 nt) comigrates with the product protected by the actin probe (250 nt) and therefore cannot be quantitated within the same lane. However, transcripts from the endogenous and transfected *Wnt-4* genes were quantitated in lane 26 of Fig. 4.

ing that these individual members of the *Wnt* gene family are involved in normal growth regulation in this tissue.

Both the marked patterns of expression of the *Wnt* genes during mammary development and differentiation as well as the differential ability of these genes to cause transformation of mammary epithelial cells (Table 3) suggest that certain *Wnt* genes may be specialized for either proliferative growth or differentiation. Alternate roles in growth and differentiation have been shown for other peptide growth factors which act on mammary epithelia throughout postnatal development. Insulin stimulates cell division during pregnancy and lactation (34) and potentiates the lactogenic effects of prolactin (2), and high-affinity insulin receptors are expressed at elevated levels in early-pregnancy mammary gland (16). Epidermal growth factor performs a dual function, stimulating epithelial cell proliferation while inhibiting functional differentiation (48, 49; reviewed in reference 3). Epidermal growth factor may also inhibit ductal morphogenesis (8). Transforming growth factor α (TGF-α) stimulates both ductal and alveolar growth (54). In transgenic mice, the overexpression of TGF-α in the mammary gland causes epithelial hyperplasia, adenocarcinoma development, and aberrant mammary gland morphogenesis (17, 23, 43). TGF-β has an inhibitory effect on the growth of mammary gland ducts (9), and its expression is induced by tamoxifen (38). TGF-β has also been shown to induce expression of a marker of differentiated mammary epithelial cells, in both normal and oncogene-transformed human mammary epithelial cells (56), suggesting that this peptide growth factor may play a role in the induction of differentiation. Thus, a role for stimulation of growth and differentiation has been demonstrated for a variety of peptide growth factors affecting mammary epithelial cells. It seems likely that the *Wnt* genes, which are expressed at different stages of mammary gland development, may also have different effects on epithelial cells in the mammary gland.

TABLE 3. Summary of *Wnt* gene expression and transformation potential

Gene	Endogenous expression in[a]:		Morphological transformation of C57MG cells[b]
	Mammary gland	C57MG cells	
Wnt-1	−	−	++
Wnt-3A	−	−	++
Wnt-4	++ (virgin) ++ (early preg.)	+	−
Wnt-5A	+ (early preg.)	+	−
Wnt-5B	++ (mid-preg.)	−	+
Wnt-6	++ (late preg.)	−	+
Wnt-7A	−	−	++
Wnt-7B	++ (virgin) + (early preg.)	−	+

[a] *Wnt* gene expression in the mammary gland was previously reported by Gavin and McMahon (11). *Wnt* gene expression in C57MG cells was previously reported by Gavin and McMahon (11) and is shown in Fig. 4. −, nondetectable expression; +, low-level transcription detected; ++, high-level transcription detected. preg., pregnancy.

[b] Transformation potential is demonstrated in Table 1 and Fig. 2. −, nontransforming; +, low-level transformation potential; ++, high-level transformation potential.

Although the putative receptors for the Wnt proteins have not been identified, there is an increasing amount of experimental data supporting one hypothesis of the signalling pathway through which the *Wnt* genes act in mammary epithelial cells. Through analysis of the embryonic mutations in *Drosophila melanogaster*, a segment polarity gene, *armadillo* (*arm*), has been identified and found to confer a phenotype almost identical to that conferred by *wg* (19, 57). However, while *wg* appears to behave in a non-cell autonomous fashion (29), *arm* appears to be a cell autonomous mutation (13, 58). This finding has led to the suggestion that *arm* acts downstream of the *wg* signal and that *arm* may be important in the perception or interpretation of or response to the *wg* signal. Recently the *arm* gene has been shown to be related to β-catenin (24), one of three cytoplasmic proteins complexing with cadherins (36). Cadherins are Ca^{2+}-dependent cell adhesion molecules which have been shown to be critical in a wide variety of steps during embryogenesis (reviewed in reference 47). One member of the cadherin superfamily is desmoglein I, a major glycoprotein component of desmosomes (15, 20), which has been shown to interact with plakoglobin (21). Interestingly, besides the similarities of the *arm* and *wg* phenotypes in *D. melanogaster*, it has also been shown that the microinjection of mouse *Wnt-1* RNA in *Xenopus* embryos causes an increase in gap junctional communication (35). Thus, it is possible that the *Wnt* genes act through a signalling pathway which involves cadherin-catenin complexes and elicits morphogenic changes in cells and tissues through this mechanism. Recently, a β-catenin homolog, p120, was identified as a novel substrate for protein tyrosine kinase receptors such as epidermal growth factor, platelet-derived growth factor, and colony-stimulating factor 1 receptors (39), suggesting that the cadherin-catenin complex may be involved in active signal transduction pathways. It has yet to be determined if this substrate or related proteins are phosphorylated in response to *Wnt* gene function in mammary epithelial cells and if this effect may play a role in the transforming effects of highly transforming *Wnt* genes.

The studies presented here have used mammary epithelial cell transformation as a biological assay for Wnt activity. These experiments have shown that the *Wnt* genes are not all alike in their effects on cells despite the high degree of structural homology among family members. A second assay of *Wnt* gene

function is based on the observation that ectopic expression of either mouse *Wnt-1* or *Xenopus Wnt-3A* and *Wnt-8* in *Xenopus* embryos causes anterior duplications of the embryonic axis (27, 44, 45, 59). In contrast, ectopic *Wnt-4* expression gives no phenotype (25), whereas ectopic *Wnt-5A* expression perturbs gastrulation but does not result in axial duplications (28). Moreover, in these experiments, *Wnt-1*, *-3A* and *-8* increase gap junctional permeability in 32-cell-stage embryos, while there is no similar increase following injection of *Wnt-5A* RNA (35). Thus, *Wnt-1*, *-3A* and *-8* appear to have activities distinct from those of *Wnt-4* and *Wnt-5A*, in good agreement with the data presented here from a quite different assay. Therefore, it seems likely that Wnt proteins fall into a few groups with divergent activities, which are likely to be evolutionarily conserved, at least among vertebrates.

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